HEAT TOLERANCE AND THE PERIPHERAL EFFECTS OF ANTICHOLINERGICS

ANNUAL/FINAL SUMMARY REPORT

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30 January 1988

Supported by

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-83-C-3181

Departments of Medicine (Dermatology) and Environmental Health
University of Washington
Seattle, Washington 98195

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Washington; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Seattle WA 98195	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-83-C-3181
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO. 3M1-	TASK NO.	WORK UNIT ACCESSION NO.
62734A	62734A875	AG	443

11. TITLE (Include Security Classification)

Heat Tolerance and the Peripheral Effects of Anticholinergics

12. PERSONAL AUTHOR(S)

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13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual/Final Report	FROM 83/9/1 TO 85/12/31	1988 January 30	34

16. SUPPLEMENTARY NOTATION

The Annual covers period Sept. 1, 1984-December 31, 1985. The Final covers period September 1, 1983-December 31, 1985.

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	iontophoresis, cholinergic assay, sweat glands, RAV
06	15		
06	19		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

A non-traumatic technique was devised for graded stimulation and inhibition of eccrine sweat glands with the cholinergic agonist acetyl-beta-methacholine Cl (MCh) and the antagonists atropine sulfate (AtS), atropine methylbromide (AtMeBr) and scopolamine hydrobromide (ScHyBr) by iontophoresis. Dose was adjusted using a constant cation flux (0.13 mA-cm^{-2} for 180 s) and 8 log-spaced ratios of drug cation (.16% to 100%) to Na^+ (99.84% to 0%) in 20 mM solutions of drug + NaCl. Doses were applied, 4 at a time, onto 2.1 cm^2 circular forearm sites spaced 2.6 cm apart. Drug application was followed by measurements of sweating rate per cm^2 (SR) and active gland density (AGD) at peak of drug action (about 10 min). All subjects were healthy, 18 to 30 yrs, tested while resting at 20-22°C. **GENDER**: Responses of 27 males and 12 females showed that both SR and AGD increased as a function of log MCh dose. SR was affected by gender only at the 2 highest doses (male SR slightly greater). At all but the lowest dose AGD was significantly greater for females. **ACCLIMATION**: Responses of 6 males to MCh were measured before and after

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Virginia M. Miller	301/663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

19. Abstract (continued)

acclimation for work in wet heat. AGD was unaffected at all dose levels. SR was unchanged at the 4 lower dose levels but was increased at the higher 4 levels, supporting the hypothesis that acclimation amplifies the secretory response to transmitter.
ANTICHOLINERGICS: In 12 males areas were first treated with NaCl, then with graded doses of AtS, AtMeBr or ScHyBr, then with 100% MCh. Responses at each dose level were compared with response to 100% MCh on a control area. Decline in SR, as percent of control response, was linearly related to log antagonist dose. ED_{50} potency ratios of AtS:ScHyBr:AtMeBr were
1:4-1/2:18.

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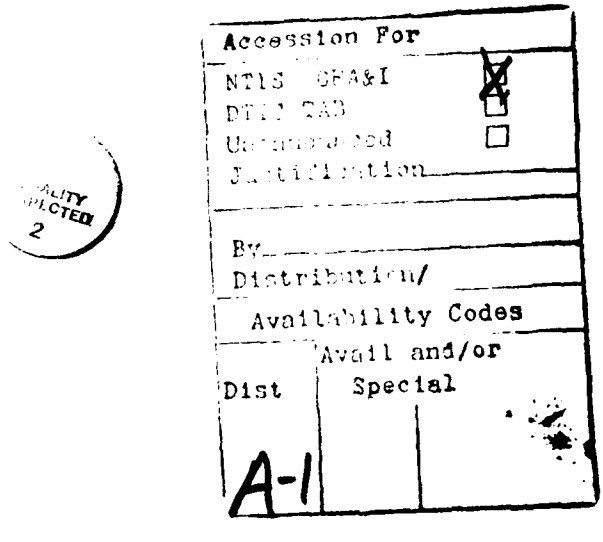

FOREWORD

For the protection of human subjects, the investigators have adhered to policies of applicable Federal Law 45CFR46.

These studies were approved by the University of Washington Human Subjects Review Committee.

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INTRODUCTION

In clinical studies of anticholinergic efficacy in humans in which either basal level of parasympathetic tone or heat stress is used as a stimulus, it is difficult to estimate potency directly because the acetylcholine (ACh) level being antagonized is unknown. Clinical studies are further confounded by questions of metabolism, bioavailability, and pharmacokinetics [1-8]. Direct potencies of cholinergic agonists and antagonists are often determined from *in vitro* dose-response assays on isolated tissues suspended in organ baths, where the concentration of drugs can be carefully controlled [1,9,10]. Yet because there are no animal models that can substitute perfectly for human responses, studies on human subjects must always form the bases for decisions about dosage and drug of choice [1,11,12]. It would be useful, therefore, to supplement findings of conventional clinical studies with data from a direct human assay method that would have the precision of the organ bath but could be used *in situ* on a sensitive muscarinic end organ. Requirements for a direct human cholinergic dose-response assay are (1) that it can be used safely *in vivo* without pain or aftereffects, (2) that it be quantitative, and (3) that it measure responses of agonists and antagonists applied directly to an accessible muscarinic end organ, without metabolic or pharmacokinetic complications. Further, the muscarinic end organ (4) should not have substantial basal activity, as this could interfere with the assay. Preferably, the assay would be (5) noninvasive and (6) suitable for application in screening studies of normal subjects or clinical studies of outpatients. A direct assay might also be useful for obtaining directly relevant human data on specific, applied problems, such as those encountered in military medicine, that are too difficult or hazardous to obtain by other experimental approaches.

The eccrine glands seem an ideal *in vivo* end organ system for direct cholinergic assay. They constitute a dense (150-300 cm^{-2}), distributed-organ network over the general skin surface. Although they are innervated by postganglionic fibers of the sympathetic nervous system, their active neuroglandular unions appear to be predominantly cholinergic and muscarinic [1,13]. Sweat glands are accessible for stimulation and measurement from the skin surface. Studies *in vitro* have well characterized the responses of isolated human glands to cholinergic agonists [9]. It has been shown that sweat gland activity is at least as sensitive as that of salivary glands to the effects of systemic atropine sulfate and is apparently much more sensitive than other non-invasive measures, such as heart rate, pupil size, and near-point visual accommodation [11].

Clubley *et al.* first proposed an *in vivo* sweat gland assay [11]. They found that measurement of forearm sweat responses to graded intradermal injections of ACh was a sensitive index of the anticholinergic action of systemically administered atropine sulfate. However, their assay used intradermal injection, an invasive procedure that stimulates only a small area whose size and shape are difficult to control and whose depth is difficult to reproduce. Their assay provides no method for directly administering antagonists to the sweat glands. Its only measurement is a total count of the number of glands responding, including those lying at and beyond the borders of the stimulated area: Secretion rate is not determined.

Independently, we also set out to develop an *in vivo* assay method based on the responsiveness of the eccrine sweat glands [14]. Our approach is more biophysical

than that of Clubley's group, which we think gives our method some significant advantages. First, ours is non-invasive. Skin areas are stimulated with cholinergic agonists rapidly, painlessly, and safely, without needle puncture or trauma, by iontophoresis (the transportation of minute quantities of cationic agonists and/or antagonists across unbroken skin, using the force of a weak DC electric field). Stimulated skin areas are well defined with this technique, and the stimulated area is larger than the measurement area so that stimulation border effects do not influence the data. Second, we can administer antagonists as well as agonists by iontophoresis in order to directly measure their inhibitory action on sweat gland responses. Third, we use more sophisticated measures: The sweating rate, derived from an assembly of 4 evaporative capsules with specially designed electronics, is our primary measurement. Sweat counts are determined per cm^2 of skin with iodinated paper, which has advantages over plastic impression material for quantitation and storage [15].

Development of this assay was the main focus of the first year's contract work. A major problem was that iontophoresis had previously been used only as a qualitative tool for stimulating or blocking the sweat glands [13,16,17]. A theoretical basis for quantitation of the method exists, however, and we judged that through further study a sufficiently accurate way to implement quantitative iontophoretic drug delivery could be found.

BACKGROUND

A safe, non-invasive, human assay of eccrine sweat gland responsiveness to muscarinic agonists and antagonists can be applied to problem areas of direct relevance to military medicine. This section addresses these applications.

Anticholinergic Assays

Poisoning by insecticides or chemical agents having anticholinesterase activity is usually treated with compounds that inhibit the activity of ACh on muscarinic cholinergic effector sites: Atropine sulfate is the prototype [1]. In evaluating the antimuscarinic properties of various compounds, a number of factors must be considered, including their pharmacokinetics and bioavailability. Of equal importance is a comparison of the ability of antagonists to inhibit the action of muscarinic agonists once they reach the target tissues. It has been customary to make such comparisons on animal tissue in vitro. The degree to which graded stimulation of a sample tissue by muscarinic agonist is inhibited by different doses of antagonists is often presented as a family of log dose-response plots from which estimates can be made of threshold, sensitivity, and potency [1,9]. Such analyses are important for developing new antidotal compounds and for studying their mechanisms of action. Ultimately, however, the direct relevance of animal studies to humans always must be demonstrated, and large differences in treatment efficacy have been observed between animal species because of non-specific binding of drug to tissues [12]. Thus, a safe and sensitive human muscarinic assay would be a very useful addition to the existing battery of bioassay techniques. The rate of sweat production by the eccrine glands on non-glabrous skin regions of humans is highly sensitive to the actions of muscarinic agonists and antagonists; depression of stimulated sweating can be used, therefore, as a sensitive indicator of the peripheral antimuscarinic activity in antidotes to anticholinesterases [13]. Several previous studies

have used heat stress to stimulate sweating in volunteers and measured the depression in sweating induced by systemically administered atropine sulfate as an index of antimuscarinic activity [4-8]. But these kinds of experiments are extremely limited in the amount of data that can be obtained from each subject and are expensive and time-consuming. Development of standard iontophoretic techniques for stimulating glands on small skin areas with muscarinic agonists and, at the same time, blocking this action with antagonists (administered either systemically in staircase fashion or by iontophoresis pretreatment of the test site) would allow multiple dose-response comparisons to be made safely among different anticholinergic substances on the same subject.

Thermoregulation

The primary physiological function of the eccrine sweat glands in humans is to provide a means for heat loss by evaporation; in fact, they provide the only significant physiological defense against heat stress in jungle and desert climates. The possibility of untimely administration of chemical agent antidotes cannot be ruled out. If they are injudiciously administered under conditions of jungle or desert warfare, even a well-acclimated soldier's performance could be severely compromised by hyperthermia; for the less fortunate, fatal heat stroke could result. Quantitation of decrements in heat tolerance caused by chemical agent antidotes in different doses is a long-standing problem of military medicine [2-8], but data are sparse because of the inherent difficulties in administering high doses of antidotes to heat-stressed subjects. A non-invasive assay of sweat gland responsiveness to graded muscarinic stimulation by iontophoresis, performed in resting subjects given staircase doses of antagonist, would be a way of obtaining a family of dose-response curves from each volunteer. The assay could be done in the safety of a room temperature environment.

Heat Tolerance

Heat-training protocols have often been proposed for rapidly acclimating workers and military personnel in temperate zones who must be transported abroad for heavy work or combat in tropical or desert climates. Administration of supervised heat-training protocols is expensive and time-consuming, and some persons will benefit little either because they are heat-intolerant, i.e., more prone to heat stroke, or because they are already well acclimated. A rapid method for screening and grading the heat tolerance of large numbers of persons would be beneficial in these situations, especially for identifying those who are heat-intolerant. Shvartz et al. [18] have proposed an empirical room-temperature exercise protocol for indexing heat tolerance, but this procedure requires constant supervision and measurement of heart rate and rectal temperatures. Others have proposed that sweating rate may be related to heat tolerance [19], and Collins et al. demonstrated a promising method, not further pursued, that related state of acclimation to the maximal responsiveness of sweat glands to local cholinergic stimulation [20]. Again, the assay of sweat gland responsiveness to muscarinic stimulation by iontophoresis would be a way of obtaining complete dose-response data from each volunteer. If these data correlate well to heat tolerance as determined by more conventional thermal tests, it may be possible to develop a simple screening test based solely on a volunteer's sweat responses to graded muscarinic stimulation.

RATIONALE

We had developed instrumentation for quantitating the sweating responses of several small skin areas simultaneously treated by single-dose iontophoresis, and thought that it might be possible to apply these techniques to the above problems. Although theory suggested that iontophoresis could be developed into a variable dose method, i.e., that there is a relationship between the parameters of iontophoresis (current and time) and the dose delivered into the skin, experimental documentation for this relationship has been lacking. The purpose of these studies were to develop iontophoresis as an investigative tool and to apply the techniques in demonstration projects in each of the three areas outlined above.

OBJECTIVES

The overall objectives of this study are: (1) to develop technology for obtaining accurate and reproducible dose-response data non-invasively from the eccrine sweat gland system, (2) to compare effectiveness of different locally applied cholinergic muscarinic antagonists in altering dose-response patterns of eccrine glands to locally applied cholinergic agonists, (3) to study the time course of changes in cholinergic sensitivity of the eccrine glands during acclimation to heat, and (4) to compare the effect of locally and systemically applied antagonists on responses to locally applied agonist.

METHODS

Iontophoresis

Regions. Our instrumentation (Figure 1) was designed to study simultaneously 4 adjacent treatment sites on regions of the proximal volar surface of the forearm. The proximal half shows less variance, and it is difficult to secure intimate contact of apparatus over the tendonous areas adjacent to the wrist. The test sites are of circles 2.1 cm diameter in a line with centers 2.6 cm apart. The 4-site region under study is defined by a rectangular plastic template secured by Velcro straps around the forearm. The proximal volar surface of the average male supinated forearm is large enough to define 2 side-by-side regions on each forearm without overlap. This provides as many as 16 assay sites per volunteer during a study session. Slight flexion or rotation of the forearm will cause shifts in the skin surface underlying the template; alignment should be checked frequently. In most female subjects only 1 region per forearm can be defined.

Apparatus. The anode assembly consists of a machined 11.5 cm x 3.7 cm Plexiglas block containing four 0.9 cm D Ag/AgCl buttons removed from disposable ECG monitoring electrodes. Glass fiber filter pads of 2.1 cm diameter holding 0.22 ml of pretreatment or treatment solution, sit snugly over the anode buttons in machined wells of 2.1 cm diameter and 0.1 cm depth. Greater volumes will cause leakage of the conducting electrolyte between adjacent anodes. The original anodes were copper and resulted in uneven sweat stimulation, as evidenced by sweat prints. This problem was virtually eliminated by changing to Ag/AgCl anodes. The cathode assembly consists of 4 ECG electrodes mounted

on the forearm extensor surface, parallel to and directly opposite the test sites. Each anode and each cathode are connected to independent battery-operated power sources (Figure 2) that maintain a constant current flux despite changes in resistance occurring because of polarization or hydration of the skin surface. Completely independent power sources and cathodes reduce the probability of significant interanodal currents.

Solutions. Stock solutions of NaCl and acetyl-β-methacholine (mecholyl) are prepared at a concentration of 20 mM in twice-distilled, deionized, bacteria-free water (Sigma Chemical Company, St. Louis, MO 63178). Measured amounts of drug stock solution are diluted with 20 mM NaCl stock solution (Figure 3) to prepare a series of pretreatment or treatment solutions in which the total ion concentration is the same (20 mM) but the relative proportion of active drug ion is adjusted from 0.1% to 100%. From threshold to maximal sweating rate requires a 10^3 to 10^4 increase in agonist dose [9,21]. Changes in iontophoresis current cannot span this dose range. With the "ratio method," drug dilutions with NaCl can be made quite accurately over a 10^4-fold range.

Calculations. From Faraday's Law of Electrolysis, the molar quantity of cationic material transported across the pad-skin boundary is proportional to the total charge moved (current x time) and to the ratio of drug to total ions in the solution-soaked pad. This quantity is given by:

$$Q = (\alpha \cdot I \cdot t)/(z \cdot F)$$

where Q is the predicted quantity of drug cations transported in nMol-cm^{-2}, α is the molar fraction of drug ions in the iontophoresis solution, I is the current density in mA-cm^{-2}, t is the duration of iontophoresis in seconds, z is the charge number of the cation, and F is Faraday's constant ($9.65 \cdot 10^{-2}$ mA-sec-nMol^{-1}). In both the dose-response tests and antagonist pretreatments, I and t are first chosen so that the highest desired dose will just be obtained using pure drug solution, i.e., $\alpha = 1.0$. The same values for I and t are used on all sites. Lower doses are obtained by using the solutions with smaller α values (Figure 3).

Procedures

Pretreatment. If agonist iontophoresis is attempted on unconditioned skin, the sweat glands often are not stimulated as expected. This problem has been noted by others and may occur because the skin, initially dry, exhibits an extremely high electrical resistance [22,23]. We found that agonist iontophoresis is optimized by a "conditioning" pretreatment: iontophoresis of the same sites with 20 mM NaCl solution alone. By hydrating the stratum corneum, pretreatment reduces the electrical resistance and permits appropriate current levels to be maintained with low voltages during subsequent agonist iontophoresis. Pilot tests showed that NaCl pretreatment at 0.06 mA-cm^{-2} for 240 sec produces an adequate conditioning effect. Simply moistening the skin with electrolyte is not as effective as iontophoresis.

Treatment. Following pretreatment, the skin area inside the template is blotted dry and the iontophoresis procedure is again applied to the same sites, using dilutions of mecholyl. In both the pretreatment and treatment stages, the same procedure is followed: The anode assembly is placed into the

template and the currents are rapidly adjusted to the prescribed level and held at this level for the prescribed time. We have used current densities from 0.06 to 0.15 mA-cm^{-2} and durations from 30 to 360 sec.

Sweat Measurements

Sweating Rate. Immediately after agonist iontophoresis, 4 small circular evaporative sweat capsules whose centers align with the treatment centers are placed on the sites and secured to Velcro fasteners on the template guide. The skin area exposed inside each capsule is 2.85 cm^2, purposely smaller than the treated area, to eliminate border effects of untreated skin. The sweat capsules use fast-responding capacitance relative humidity sensors (Figure 4); details of their design and construction are fully described elsewhere [15]. Briefly, dry nitrogen gas at 200 ml-min^{-1} is directed uniformly onto the skin surface inside each capsule. At 200 ml-min^{-1}, the capsule environment is completely exchanged every 2.3 sec. Initial tests showed this rate to be adequate for complete and rapid evaporation. The effluent nitrogen and evaporated water vapor mixture then passes into an upper chamber containing relative humidity and temperature sensors. Analog voltages corresponding to the measured relative humidity and temperature are automatically digitized every 5 sec and stored by a microcomputer on disk for future detailed analysis. Data are also processed on-line and displayed on a video monitor. Measurements of baseline transepidermal water loss are obtained before the start of the experiment. These minor corrections (0.01 to 0.03 mg-cm^{-2}-min^{-1}) are automatically subtracted by the computer from succeeding readings.

Active Gland Density. In most experiments the sweat capsules are removed soon after peak sweat rates are achieved, the skin is quickly blotted with tissue, and an impression of active glands is made using iodized paper. Dessicated xerography paper is cut into 1- by 6-inch strips and hung for 24 to 36 hr in a chamber containing a few grams of iodine crystals in an open dish. Prepared strips are fastened onto the holder (Figure 1), and steady and even pressure is applied for 10 to 20 sec, depending on the sweat rate. Paper starch reacts with iodine wherever the two are solubilized by a sweat droplet. prints will keep for years if stored away from light in plastic 35 mm negative sleeves. Subsequently, assessment of active glands under each capsule area is made with the aid of a low-power surface microscope, a calibrated grid that subdivides the capsule area, and a computer program that tallies the grid counts. Grouped data in Figures are presented as average and SEM.

Acclimation Protocol

Step Test. The step test was conducted in the 23°C laboratory environment and consisted of 15 min of stepping from the floor to a height of 30 cm at a rate of 50 steps/min. Before the step test, subjects were outfitted with a photoelectric earlobe sensor that connected to an external cardiotachometer, a thermocouple rectal probe (10 cm) sensitive to 0.01 C, a forehead evaporative sweat capsule and forehead skin temperature thermocouple inside the capsule. The skin temperature thermocouple often failed, and this measurement had to be discarded. Prior to the step test, subjects sat until stable resting values for heart rate and rectal temperature were obtained.

Bath Test and Acclimation Procedure. The bath test was conducted in a

specially designed 2' x 6' x 4' stainless-steel tank containing a low chair and a bicycle ergometer that was modified to permit pedaling with the legs in a horizontal position. The tank was filled to neck level prior to each experiment with water at 38°C. This temperature was maintained by recirculating the bath water through a small, thermostatically controlled water heater at a rate of 30 gallons per min. Additional stirring was provided by a battery-operated outboard trolling motor with guarded blades. Subjects were instructed to keep their arms and hands in the water at all times. On the first and last acclimation days, the same continuous measurements were made as in the step tests. Tests were terminated if rectal temperature rose above 39°C or if heart rate was greater than 180 bpm.

Subjects

All subjects were healthy adult volunteers, mainly students, recruited by advertisement in the surrounding university community. They were informed of possible risks and they freely consented to participate in these studies. Excluded were those having any chronic disease, allergy, family history of allergy, or evidence of acute illness on the day of the experiment. Records were kept of their heat stress and exercise history for future data segregation. All tests were performed in an air-conditioned laboratory with subjects in a thermally neutral state.

In addition, the 6 male subjects who participated in the acclimation experiments were given a physical examination by a licensed physician prior to their participation. All were deemed free of obvious cardiorespiratory disease and fit to cope with the severity of the procedures.

RESULTS

Example Assay Data

Data selected from 1 male subject are shown in Figure 5 to illustrate raw data obtained in the mecholyl iontophoresis assay. Typically, peak sweating rate occurs about 8 to 10 min following the end of iontophoresis. In this early experiment, iontophoresis time was 30 sec and the current was 0.058 $mA\text{-}cm^{-2}$ on all sites. Mecholyl dose was varied by adjusting α, and ranged from 0.2 to 17 $nMol\text{-}cm^{-2}$ of skin surface. For most subjects, a dose greater than 17 $nMol\text{-}cm^{-2}$ is needed to obtain peak sweating rate; in this experiment we may have failed to obtain the subject's true peak sweating rate. Our conditions are now standardized at 0.132 $mA\text{-}cm^{-2}$ and 180 sec, which gives a maximal dose of 250 $nMol\text{-}cm^{-2}$. Sweat rates, expressed as a percent of the highest sweat rate observed, vary hyperbolically with calculated dose (Figure 6a). Figure 6b, a log dose plot of the same data, demonstrates that this method of iontophoretic dose adjustment yields sigmoidal response curves expected of dose-response assays [1]. Log dose-response curves were fitted to data by Foster's least-squares method [21].

As soon as peak recordings are completed, sweat prints of the region are made (Figure 5); at the same time, capsules are transferred to the region where iontophoresis has just been completed. We define a gland as above threshold if it appears on a sweat print, and we assume the maximal active gland density to

be that observed at peak sweating rate. Sweat print counting errors are greatest at maximal sweating rate levels because of overlaps in the secretion area of 2 or more glands. Shorter imprint times are sometimes useful. Figure 6c is a normalized plot of log dose vs. percent of active glands above threshold. Over this dose range, the sweat rate and gland count curves have nearly identical shapes, although the latter has somewhat more scatter. The slope of the cumulative percent curve is bell-shaped and can be thought of as the distribution of thresholds about dose (Figure 6d). Mean threshold dose corresponds to the ED_{50}.

Regional Variation

The proximal region of the flexor forearm surface constitutes the experimental field. The capsules are small enough to allow 2 side-by-side experiments on each forearm. Thus, 16 dose-response tests can be performed in a single subject session. We attempted to determine whether variation in responses between forearm skin regions is random or consistently related to arm or to aspect. In 7 male subjects we determined responses to 1 very low and 3 very high mecholyl doses on the 4 forearm regions. Corresponding sites on the forearm regions received identical mecholyl treatments: site 1 = 1 nMol-cm^{-2}, site 2 = 100 nMol-cm^{-2}, site 3 = 160 nMol-cm^{-2}, and site 4 = 250 nMol-cm^{-2}. This protocol is now standard for all subjects and is performed on region A. It allows us to prejudge the dose yielding a maximal response and to adjust the dose-response assay accordingly. Figure 7 is a plot of means and SEMs of peak sweating rate and active gland counts for the 4 regions, grouped by dose. In terms of active glands, region d was consistently, but not significantly, higher than the other 3 regions. This was not true for peak sweating rate. The 250 nMol-cm^{-2} dose (site 4 on all regions) gave more variable, but not significantly different, sweating rates compared to those of the other 3 doses. No significant differences in either peak sweat rate or active gland density were found among the 3 highest doses on any skin region. Variability was lower for both parameters at 1 nMol-cm^{-2} than at the 3 higher doses.

Current and Duration

Although Faraday's Law predicts that combinations of current and time yielding equivalent charge transfer should transport the same number of ions into the skin, the velocity of ionic migration is related not to charge transfer but to the potential drop between the source (anode) and the sweat glands. Therefore, assuming the skin is an ohmic conductor, a higher velocity would be expected at higher current levels. Thus a combination of low current and long time might produce a significantly different distribution of drug cations around the sweat glands than an equivalent charge transfer from a high current/short time combination. To study this, we compared the responses in 5 male subjects to mecholyl iontophoresis on 4 left arm sites, using 4 different time-current combinations giving the same charge transport (13.9 mC-cm^{-2}) and total dose (16 nMol-cm^{-2}). They were 0.029 mA-cm^{-2} for 480 sec, 0.058 mA-cm^{-2} for 240 sec, 0.116 mA-cm^{-2} for 120 sec, and 0.173 mA-cm^{-2} for 80 sec (Figure 8c). There appeared to be slight fall-off in peak sweating rate as current density was increased and duration was reduced, but this change was not significant (Figure 8b). Corresponding sites on the right arm were used as controls and were all treated at 0.029 mA-cm^{-2} for 120 sec. There was no trend of change in these control areas nor was there a significant difference between them

(mean = 6.45 mg-min^{-1}cm^{-2}). Time of peak response (end of iontophoresis = time zero) was inversely related to the log of iontophoresis duration (Figure 8a).

Long-term Changes

We restudied 6 male subjects a number of times over a 3-4-month period, in experiments in which different conditions of time and current were used. The pooled dose-response data are compiled for each subject in Figure 9. For the most part there is a reasonable agreement in the dose-response data over several months in spite of different currents and iontophoresis times, although some subjects showed much more variability than others. Subjects who showed the most consistent responses within an experimental session tended to show the most consistency in response among experimental sessions.

In Vitro Studies

To determine the feasibility of using in vitro studies to obtain more exact knowledge of drug transport during iontophoresis, pilot tests were done on 5 samples of human abdominal skin obtained at autopsy. Iontophoresis was performed with ^{14}C-lidocaine-HCl. Lidocaine is of comparable size and weight to mecholyl and should have the same order of electrophoretic mobility. Current density was 0.29 mA-cm^{-2} for 30 sec on 4 samples; no current was applied to the 5th sample, which served as a diffusion control. Following iontophoresis, the sample epidermis and dermis were separated by heating between two 60°C aluminum plates for 30 sec. Samples 1-4 were split at 2, 6, 10, and 20 min after iontophoresis, respectively; the control at 22 min. Epidermal and dermal samples as well as anodal filter pads containing the remaining labeled lidocaine and cathodal saline-soaked pads were placed in individual vials, digested and counted in scintillation fluid. Results, shown in Figure 10, indicate that (1) an extremely small amount of ^{14}C-lidocaine, within the limits of counting accuracy, was transferred from the top pad into the skin by simple diffusion alone (sample 5); (2) there was no trend of difference among samples 1-4, indicating insignificant diffusional transfer during the 20-min period following iontophoresis; (3) an average (samples 1-4) of only 1% of label reached the subdermal pad; (4) an average of 9% of the delivered dose reached the dermis; (5) the calculated total amount transferred was less than the observed amount; and (6) 90% of the delivered dose remained in the epidermis. Juhlin [24] observed that the "epidermal pool" of drug remains for several days after iontophoresis. Subsequent iontophoresis of NaCl solution over the test site reinduces substantial activity. We also have observed this storage, and we allow 2 to 3 weeks before retesting at the same site; this delay accounts for the normal shedding and rejuvenation cycle of the stratum corneum.

Gender Differences

One application of the mecholyl assay was to obtain comparative response data on young normal male and female subjects. A dose range of 1 to 250 nMol-cm^{-2} was studied in 12 male and 12 female subjects. An additional dose level of 0.4 nMol-cm^{-2} was tested on 6 of the female subjects. Peak sweat rates were not significantly different for males and females up to a dose of 40 nMol-cm^{-2}, after which sweat rate in males tended to be somewhat higher (Figure 11). At all doses above 1.0 nMol-cm^{-2}, female subjects showed a significantly greater

active gland density and a greater rate of change in gland density with dose than their male counterparts. This would indicate that the output per sweat gland is greater for males than for females. We consistently observed while counting that the gland spots on female sweat prints were smaller than on male sweat prints, but had no quantitative method for measuring average spot size. When data were normalized to the highest observed response and replotted, it was seen that the percentage of total glands above threshold for any given dose was essentially the same for both males and females (Figure 12). Since the total number of sweat glands is supposedly fixed and not gender-specific from birth to puberty, we hypothesized that a large part of the difference in sweat gland density between males and females is not related to gender per se but to anthropometric differences, since males generally have larger forearms than females.

To test this hypothesis, we measured forearm dimensions and calculated the total skin area at the locus of the test sites as the curved surface of the frustrum of a right cone [25]. Figure 13 shows that maximum gland density and forearm surface area at the capsule sites are related inversely.

CW Antidote Potency Assay

We investigated the possibility that the sweat gland assay could be used to rank directly and locally the relative potency of different anticholinergic substances, thus reducing the pharmokinetic and bioavailability issues that complicate systemic administration. In 12 male subjects the antagonists atropine sulfate, scopolamine hydrobromide, and atropine methylbromide were applied as pretreatment, each in multiple doses (0.5 to 250 nMol-cm^{-2}). Each site was then treated with a dose of mecholyl previously determined to produce maximal sweating rate in that subject in the absence of inhibitor (100 to 250 nMol-cm-^{2}). Mecholyl iontophoresis on these sites was further delayed by 7 min to allow the full action of the antagonist to take place. For each subject the percent inhibition of maximal sweat rate (measured on control sites) was calculated for each antagonist site. The results are plotted in Figure 14. Inhibition of sweating by the 3 antagonists was qualitatively similar but quantitatively different. The ED_{50} was 3 nMol-cm^{-2} for atropine methylbromide, 12 nMol-cm^{-2} for scopolamine hydrobromide and 54 nMol-cm^{-2} for atropine sulfate. Using atropine sulfate as the standard, the potency ratio of scopolamine hydrobromide was 4.5 and that for atropine methylbromide was 18.

Changes with Heat Acclimation

Dose-response characteristics of forearm sweat glands to local cholinergic stimulation were measured before and after acclimation for work in wet heat to determine the extent to which glandular sensitivity is altered. Six fit male subjects (mean age = 27 yr, mean VO_2max = 42 ml-kg^{-1}-min^{-1}) pedaled at a work level of 256 W, 40 to 50 min daily for 10 days while seated and immersed to the neck in a well-stirred tank of water maintained at 38°C. This procedure elevated mean rectal temperature by 1.5°C each day. Our acclimation procedure produced the classical effects of lowering pre-test rectal temperature (- 0.65°C) and heart rate (- 8 beats-min^{-1}). Before and after acclimation, our standard forearm sweat assay was administered by iontophoresis, using log-incremented doses of mecholyl. Acclimation increased peak sweating rate by 40% between dose levels of 15 and 250 nMol-cm^{-2} (Figure 15). Below this level,

acclimation produced no change in sweating rate. The number of sweat glands activated at each dose level was also unchanged by acclimation. These data support the hypothesis that acclimation amplifies the secretory response when moderate to high levels of transmitter are secreted at the neuroglandular junction.

DISCUSSION

The improvements in methodology and technique have made the non-invasive assay a sensitive, quantitative, and reproducible method. Our experience with well over 500 applications of iontophoresis in the past several years has been that the method is safe, painless, and devoid of any systemic aftereffects.

From the Faraday calculation, our range of total delivered dose is from about 0.2 to 250 $nMol\text{-}cm^{-2}$ of skin surface. Corresponding agonist concentrations at the secretory cells is unknown and depends on the biophysics of iontophoretic transport. The skin is not a homogenous medium; most of the resistance to current (ion) flow is in the outermost skin layer, the stratum corneum, which is only 20 μm thick. Even when well hydrated, the stratum corneum has a resistivity 100 to 1000 times greater than that of the underlying viable epidermis and dermis [22]. It follows from this that the voltage (and thus the migration velocity) must fall sharply in transition from stratum corneum to deeper tissues. Since the rate of mass transfer is fixed by the constant iontophoretic current, it is predicted that most ions would accumulate in the stratum corneum and only a small fraction would reach the sweat gland secretory cells. Our pilot experiment with labeled cation (^{14}C-labeled lidocaine-HCl) iontophoresis in cadaver skin confirmed this: 90% of ions remained within the epidermis and only 9 to 10% reached the dermis during iontophoresis. However, no significant diffusion occurred from epidermis to dermis following iontophoresis. More studies of this kind will be needed to determine the exact nature of the relationship between calculated dose and periglandular concentration; whether this relationship varies significantly among drugs, currents, and durations; and whether the sweat glands themselves are preferential pathways for ionic transport. Without this biophysical information, the assay results themselves must be relied upon as an indicator of method precision. The consistency of responses and small standard errors of group averages strongly indicate that periglandular concentration is a relatively constant fraction of total delivered dose. This was somewhat surprising to us because errors include variation due to subject, forearm region (Figure 7), and measurement as well as variation due to drug delivery.

The parallel between peak sweat rate and active gland density has been striking in all of our studies, although active gland density tends to reach a maximum at a somewhat lower dose than does peak sweating rate. It must be emphasized that our measurements of active gland density count only the number of glands above their respective thresholds (and the threshold of the method) and do not quantify their individual secretion rates. Given this, the calculation of average output per gland (peak sweating rate/active gland density) as a function of dose would be questionable because it would assume a normal distribution of glandular outputs. Less questionable is the estimation of mean threshold dose, which is the same as the ED_{50} on the log-dose vs. active gland density curve (Figure 6d). Because of the parallel between peak sweating rate and active

gland density, it may be possible to simplify the assay method under certain conditions by eliminating measurement of sweating rate.

CONCLUSIONS

Ion-dilution iontophoresis was used with a single set of time and current conditions to achieve quantitative delivery of cholinergic agonist to sweat glands over the 1000-fold range needed to span threshold to maximal responses. Both peak sweating rate and active gland density varied hyperbolically, related with the log of agonist dose. There was reasonable agreement in dose-response characteristics of individual subjects in experiments repeated over a period of 3 months. Subjects who showed the most consistent responses within an experiment also showed the most consistent responses between experiments.

Females tend to reach their maximal sweating rates at lower doses of agonist than males. For females, the ED_{50} was 5 nMol-cm^{-2} and for males, the ED_{50} was 10 nMol-cm^{-2}. At submaximal doses, sweating rate per unit area was the same for males and females but active gland density was 40 to 50% greater for females at most dose levels. These differences in gland density were not related to sensitivity, as the mean threshold dose was the same for both groups, but were inversely related to forearm surface area. These findings support the long-held but poorly supported belief that the number of sweat glands are the same and fixed at birth for both males and females. Adult males generally have larger forearms than adult females, so their sweat gland density is usually lower. On the other hand, male sweat glands enlarge during puberty, while those of females do not. Hence, the output per gland is greater for adult males than for adult females. In normally sized individuals these differences in gland density and gland output counterbalance so that both normally sized males and normally sized females have nearly the same sweat rate per unit area.

Antagonists were delivered by pretreatment iontophoresis and their competitive actions with agonist were quantified. We were able to directly estimate the anticholinergic potency of three substances - atropine sulfate, scopolamine hydrobromide and atropine methylbromide - by measuring the dose of pretreatment agonist required to inhibit maximal sweating rate (control sites) by 50%. When ED_{50}'s were compared to the atropine sulfate, the potency ratio was 4.5 for scopolamine hydrobromide and 18 for atropine methylbromide. Thus our assay method may be a novel and safe alternative for assessing the peripheral anticholinergic effects of CW antidotes on human subjects [2-8].

The dose-response characteristics of sweat glands to cholinergic agonist were quantified before and after heat acclimation. Although it has long been known that heat acclimation results in a peripheral modification of sweating, it has not been clear whether these changes are due to an increased release of transmitter at the neuroglandular junction or to an increased sensitivity to transmitter [16,20]. The 40% increase in sweating rate response to the same agonist dosage following acclimation to wet heat is similar to the changes reported in whole body sweating. Therefore, our result confirms the hypothesis that the peripheral adaptation of the sweating apparatus accompanying heat acclimation primarily results not from an increase in the amount of neurotransmitter released but in an increased sensitivity of the sweat apparatus to neurotransmitter.

RECOMMENDATIONS

A. Our non-invasive cholinergic assay method has proved to be both safe and quantitative. We can recommend its use to others who may wish to include such measurements in their studies provided that they (1) adhere to our specifications for electrode design, (2) use independent battery-operated constant current sources, (3) keep within our specified constraints for current density and duration (the most comparable results will be obtained if current density is fixed as 0.132 $mA\text{-}cm^{-2}$, duration at 180 sec, and dose varied by ion dilution), (4) precede treatment iontophoresis with a conditioning step, and (5) use only proximal forearm areas and skin that is free of disease, cuts, or abrasions.

B. We have consistently used mecholyl as agonist in these studies because it is less susceptible to enzymatic degradation than ACh and provides longer periods of peak sweat rate [5]. Other muscarinic agonists that are even less susceptible to enzymatic action, such as pilocarpine, bethanachol, and carbachol, may be more suitable for the assay. We recommend that these compounds be compared with mecholyl.

C. Although the results on many subjects lead us to believe that iontophoretic delivery is fairly uniform among drugs and subjects, we recommend further studies on the biophysics of iontophoresis to confirm this point. The findings of these studies could lead directly to improvements in the assay. Indirectly, they could aid other investigators who might consider using iontophoresis in studies of skin blood flow or as a means for therapeutic drug delivery. Specifically, the progress of ^{14}C-labeled compounds through samples of fresh human skin, obtained at autopsy or from amputee donors, should be monitored by frozen-section radioautography and by scintillation counting of digested horizontal sections of dermis. From these studies it should be possible to construct an empirical model of the iontophoresis process from which periglandular concentration could be predicted from delivered dose.

D. McCance and Purohit [23] demonstrated ethnic differences in the number of sweat glands responding to a single dose of pilocarpine. This finding in itself does not reveal whether there are different maximal gland densities. Ethnic differences should be restudied with the non-invasive assay to clarify this point.

FIGURES

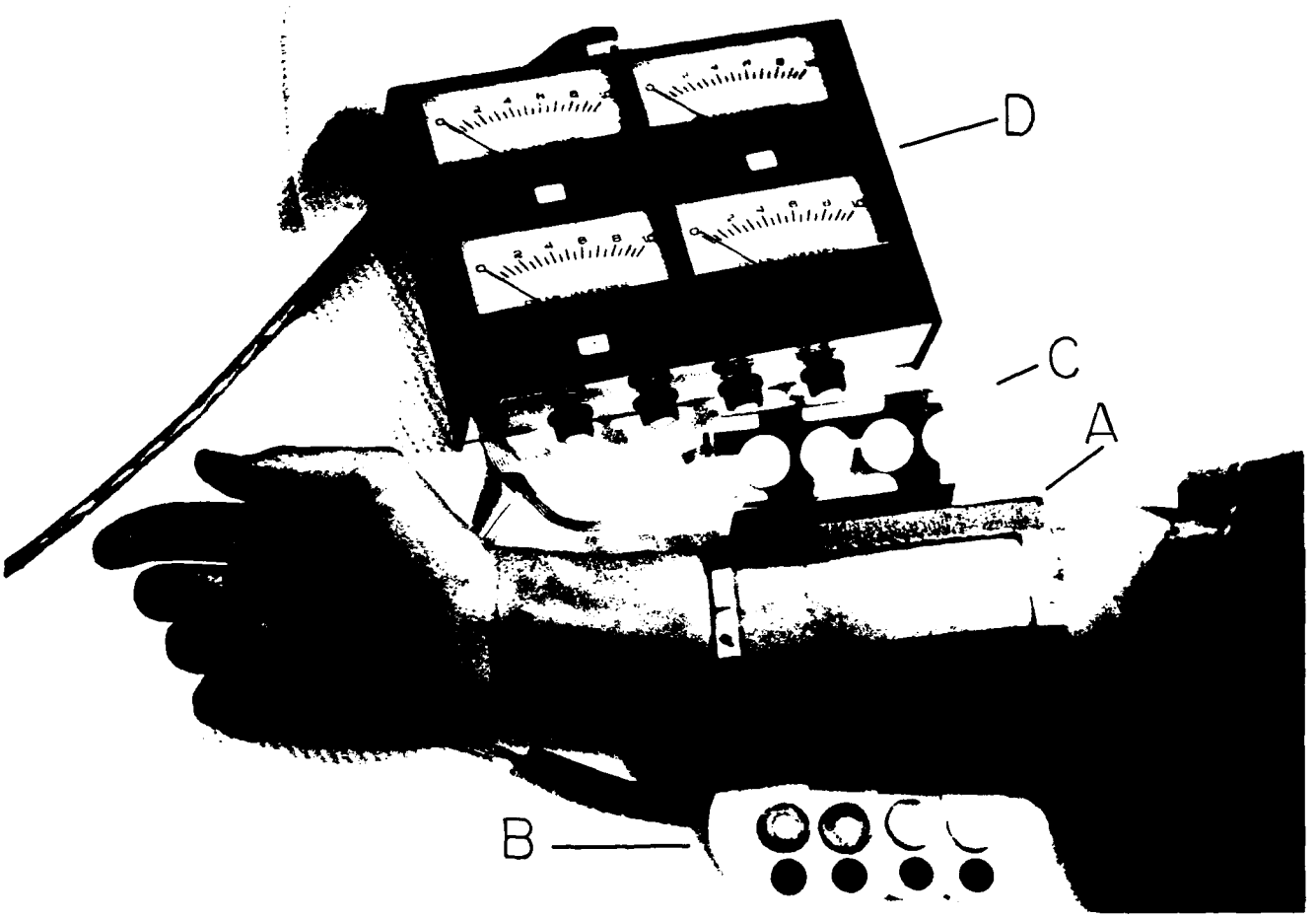

Figure 1. Iontophoresis apparatus. Test sites were 2.1 cm diameter circles 2.6 cm apart. (A) Plastic template defining the test region on flexor forearm surface. (B) Cathode assembly before attachment to extensor surface of forearm. (C) Ag/AgCl anode assembly topped with filter disks containing iontophoresis solution. (D) Control box containing 4 constant-current sources.

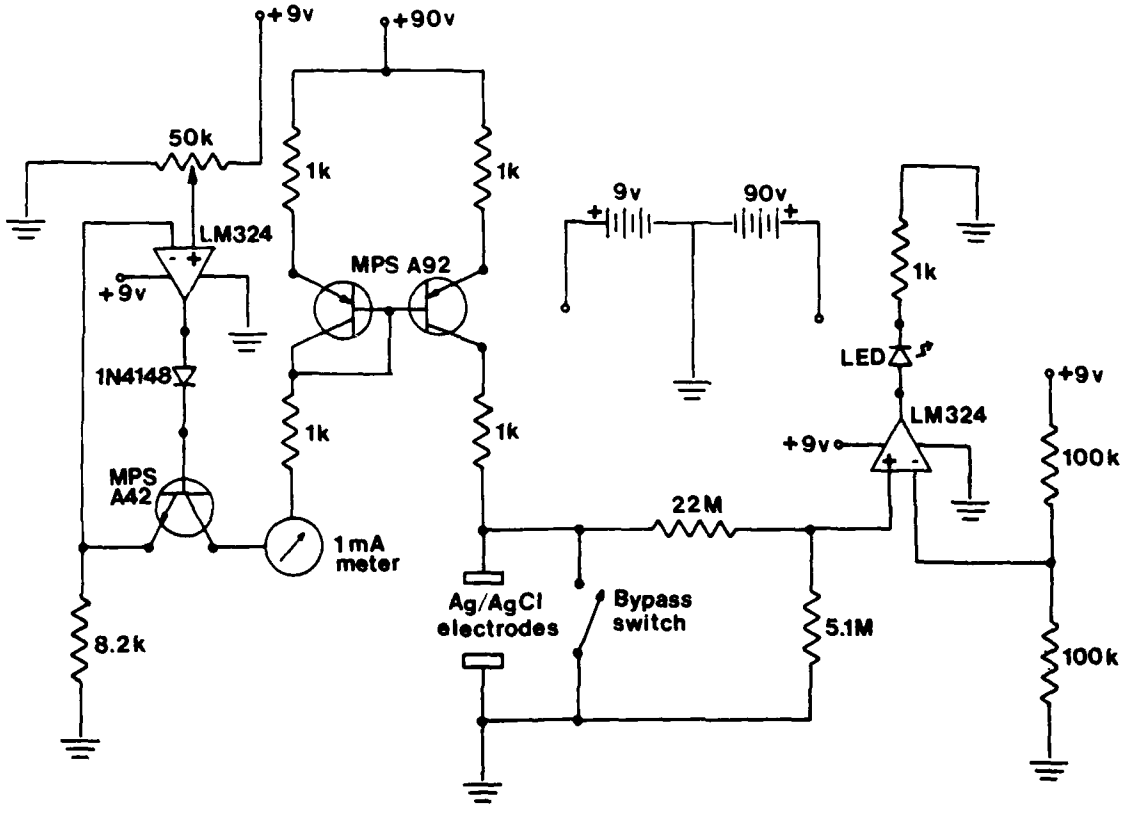

Figure 2. Schematic of constant current source driving each iontophoresis electrode pair. Power sources are 9- and 90-V batteries. Current is proportional to voltage at 50K potentiometer. Light-emitting diode (LED) is activated if voltage drop is >20 V. This occasionally occurs during the first few seconds of pretreatment. Activating bypass switch immediately brings skin potential drop to 0 V.

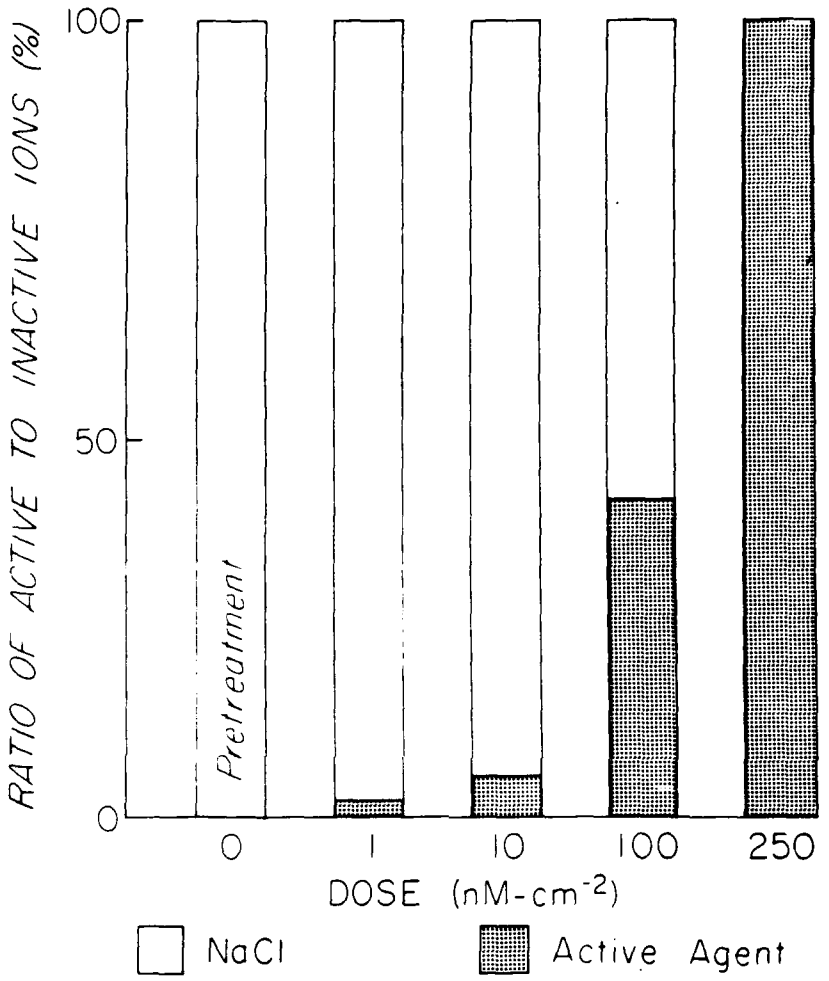

Figure 3. Adjustment of mecholyl dose by ion dilution. Using Faraday's Law, the current density and duration are back-calculated from the desired maximal dose, in this case, 250 nMol-cm^{-2}. This dose is obtained using a 20-mM iontophoresis solution containing 100% drug ions (α=1). Smaller doses are obtained by diluting this solution with 20 mM NaCl. Dose is related to percentage of charge carried by drug cations, not to absolute molarity of drug solution.

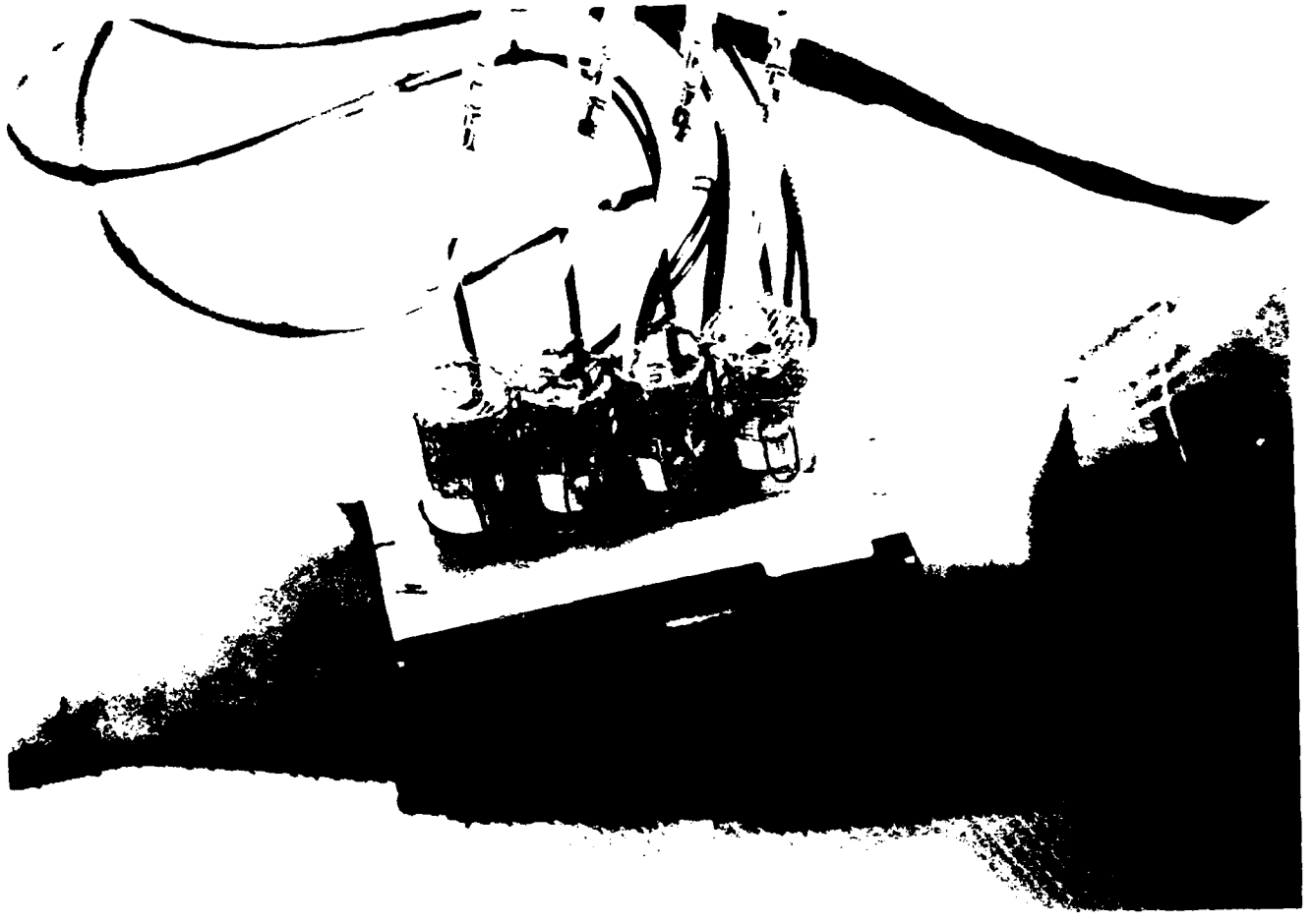

Figure 4. Evaporative capsules positioned on template during measurement of sweating rate.

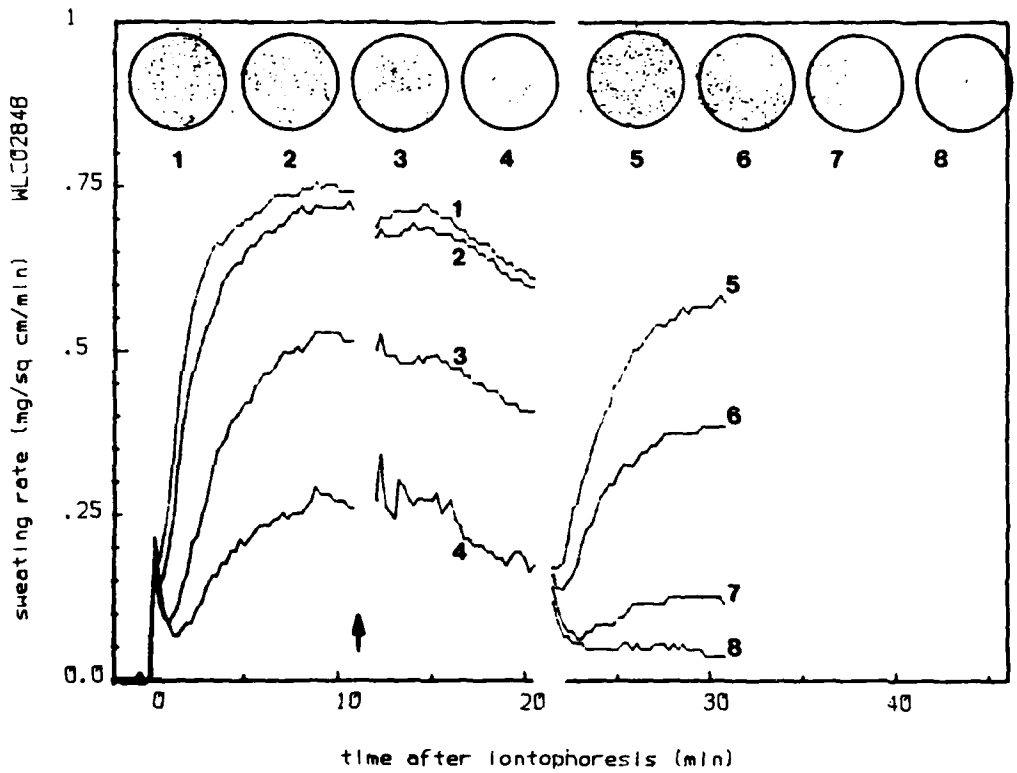

Figure 5. Computer plot of assay data on one subject. Two regions were used, one on each arm. Xerographic reproductions of sweat prints corresponding to each site appear at top of plot. Doses in nMol-cm^{-2}. Arrow indicates time of sweat prints 1-4. Capsules were then returned to sites while iontophoresis proceeded on contralateral arm.

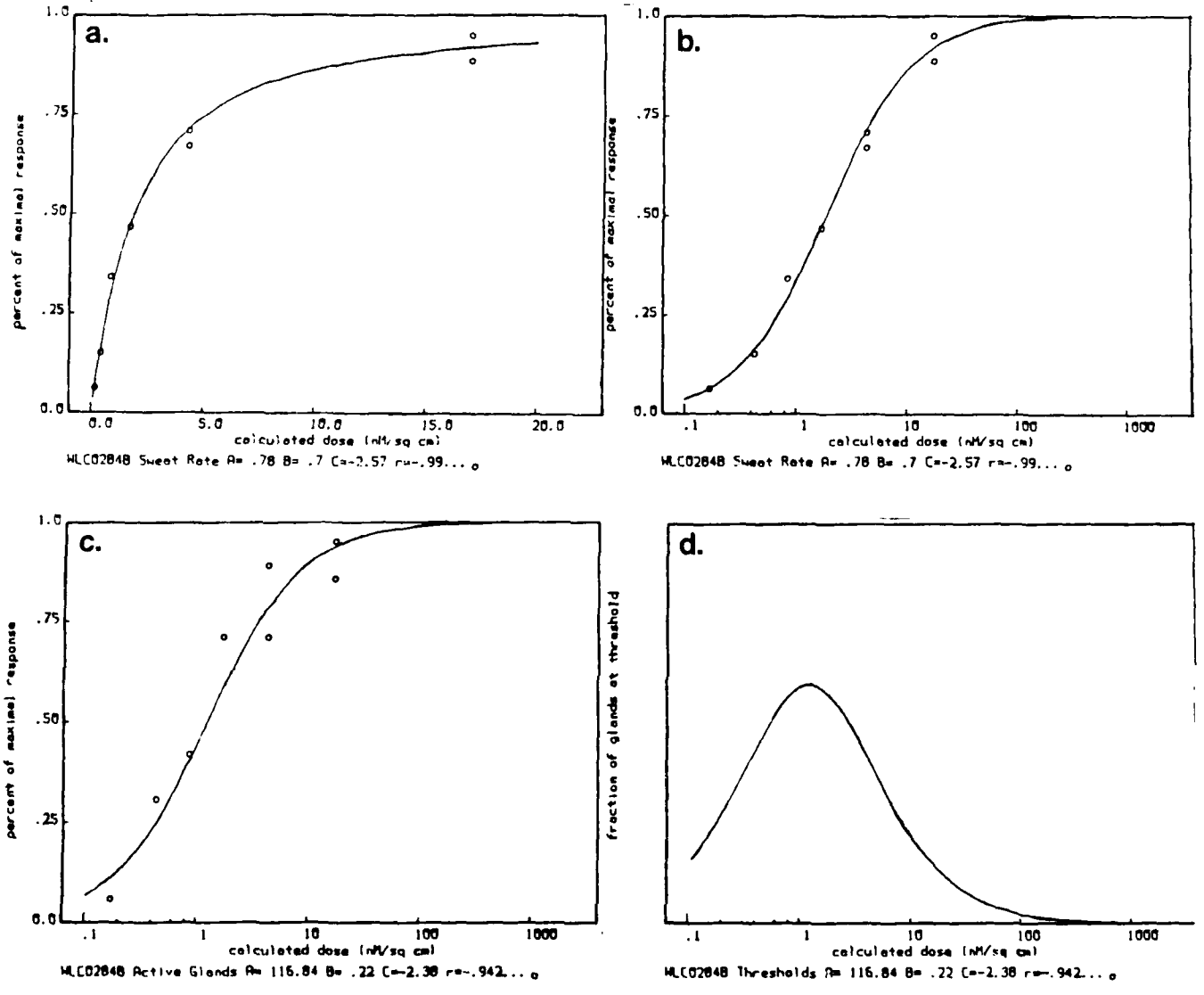

Figure 6. Peak heights and active gland densities from Figure 5 expressed as fraction of maximal response and replotted as a function of dose. Curves fit by a least-squares hyperbola. a. Peak sweating rate vs. arithmetic dose of mecholyl. b. Peak sweating rate vs. log dose. c. Active gland density (cumulative percent above threshold) vs. log dose. d. Slope of c. showing distribution of thresholds about dose. Mean threshold dose = ED_{50}.

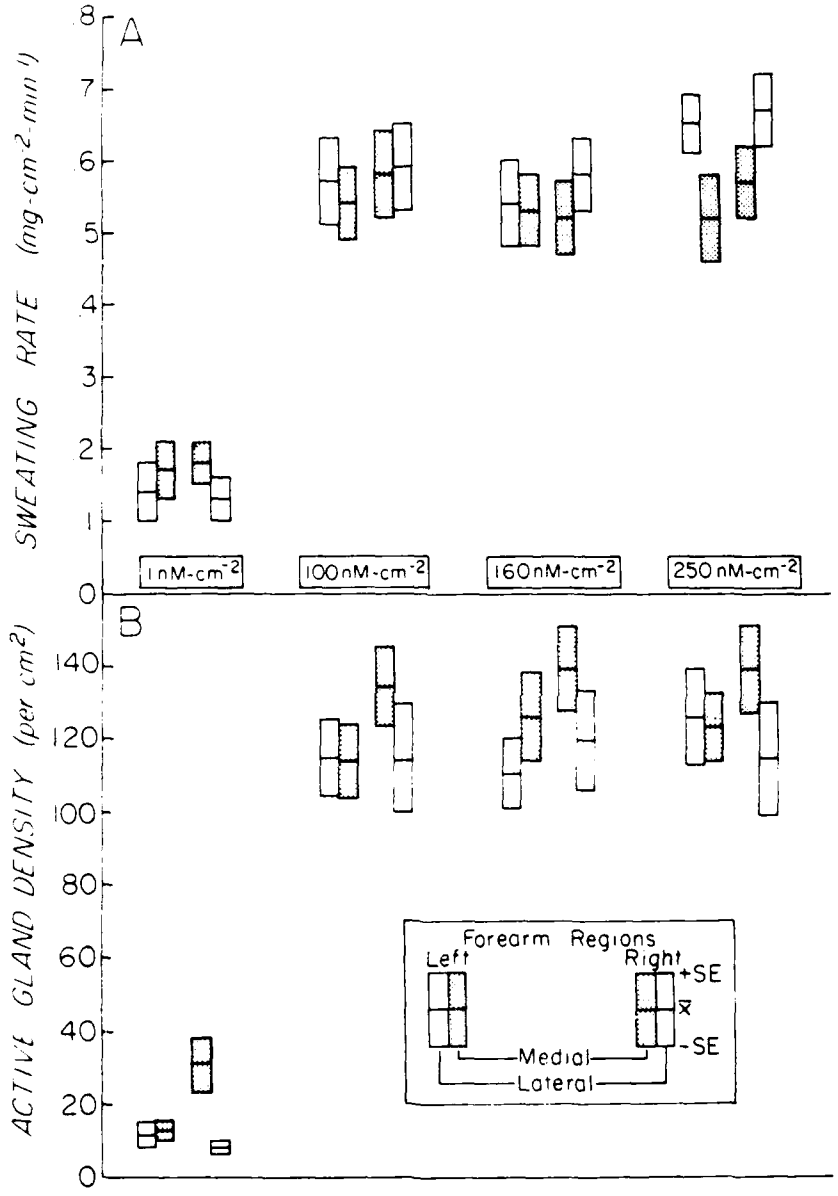

Figure 7. Regional variations in peak sweating rate and active gland density among 7 male subjects. Regions are listed as a, b, c, and d, in order of testing. Active gland density was slightly and consistently higher on region d (inner aspect of right forearm).

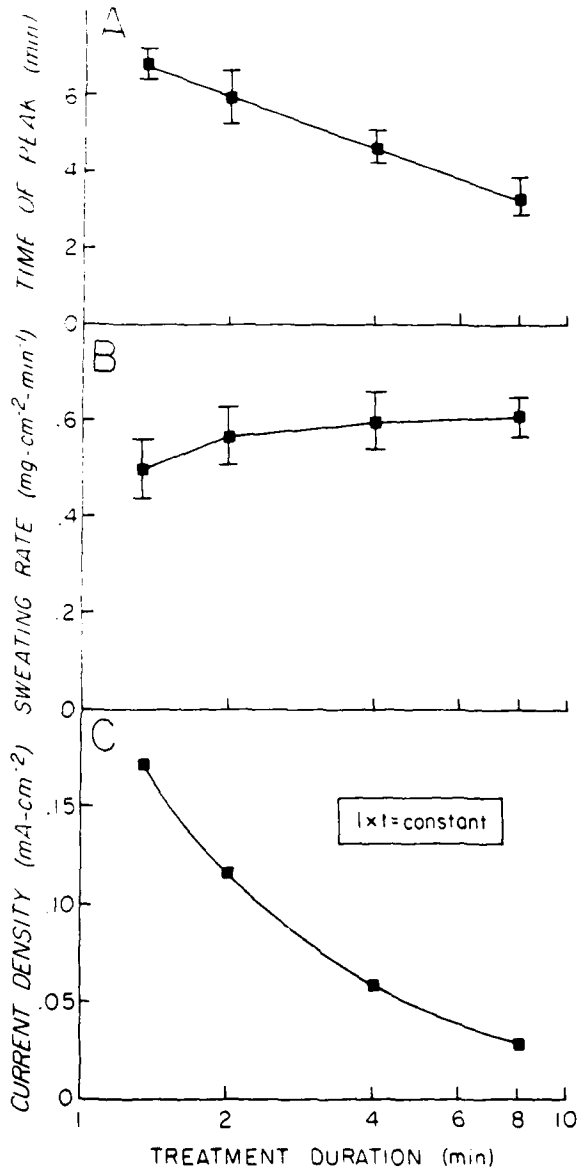

Figure 8. Effect of current and duration on delivered dose. A single midrange dilution dose of mecholyl (16 nMol-cm^{-2}) delivered by the 4 different current-duration combinations, each calculated to give a total charge transport of 13.9 mC-cm^{-2}. Height of peak response was slightly, but not significantly, lower using the highest current-shortest duration combination. Peak sweating rate time was inversely related to the log of iontophoresis duration.

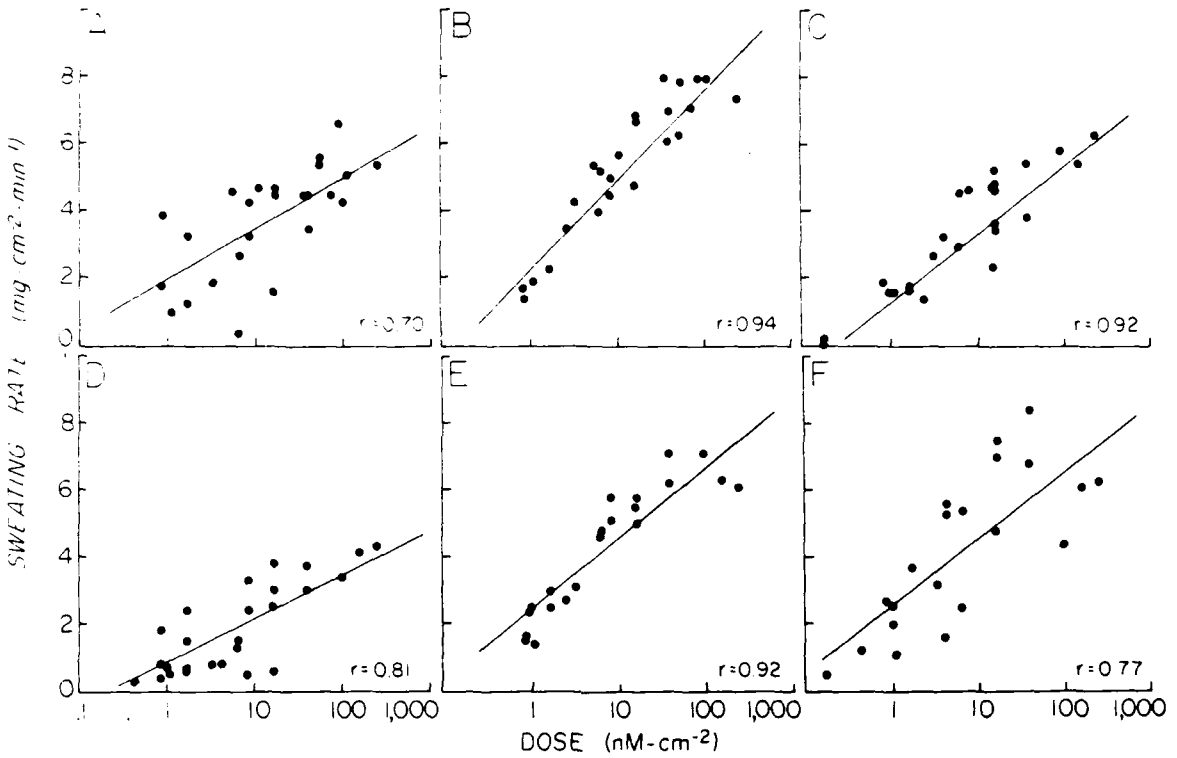

Figure 9. Repeated assays on 6 male subjects over a 3-4-month period, using several combinations of current density and duration. Subjects showing most consistent responses within an assay also showed most consistent responses between assays.

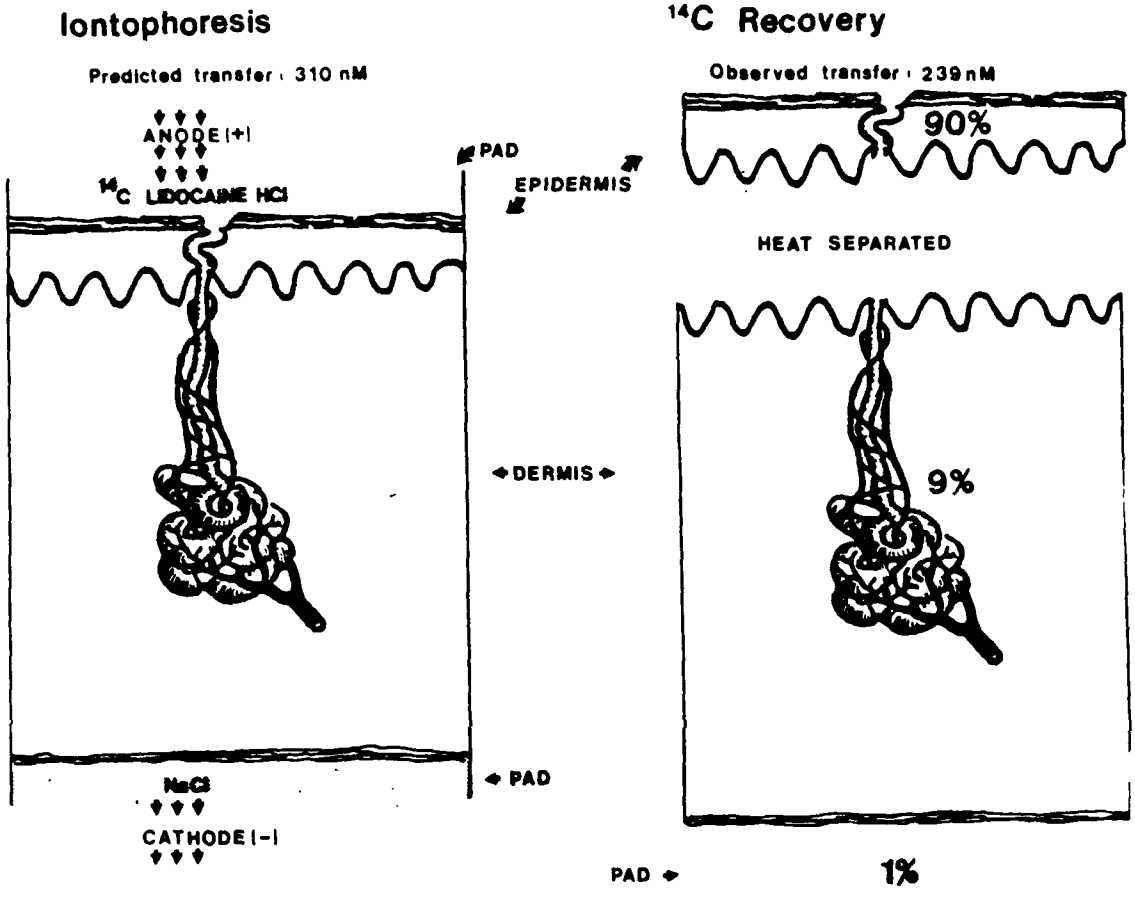

Figure 10. Results of a pilot experiment on the iontophoretic transfer of ^{14}C-labeled lidocaine-HCl through 5 samples of fresh autopsy skin. The molecular weight of lidocaine is similar to that of mecholyl, and both should have roughly the same electrophoretic mobility. Most of the label remained in the epidermis; less than 10% of the labeled cations actually reached the dermis.

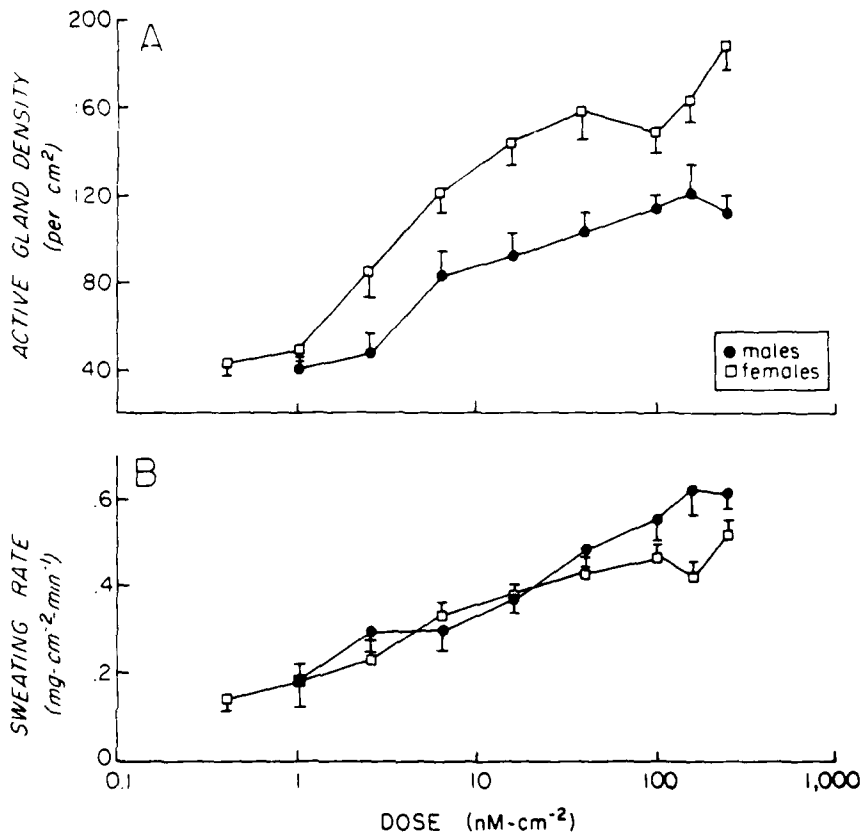

Figure 11. Peak sweating rates and active gland densities in response to the cholinergic assay in 12 normal male and 12 normal female subjects.

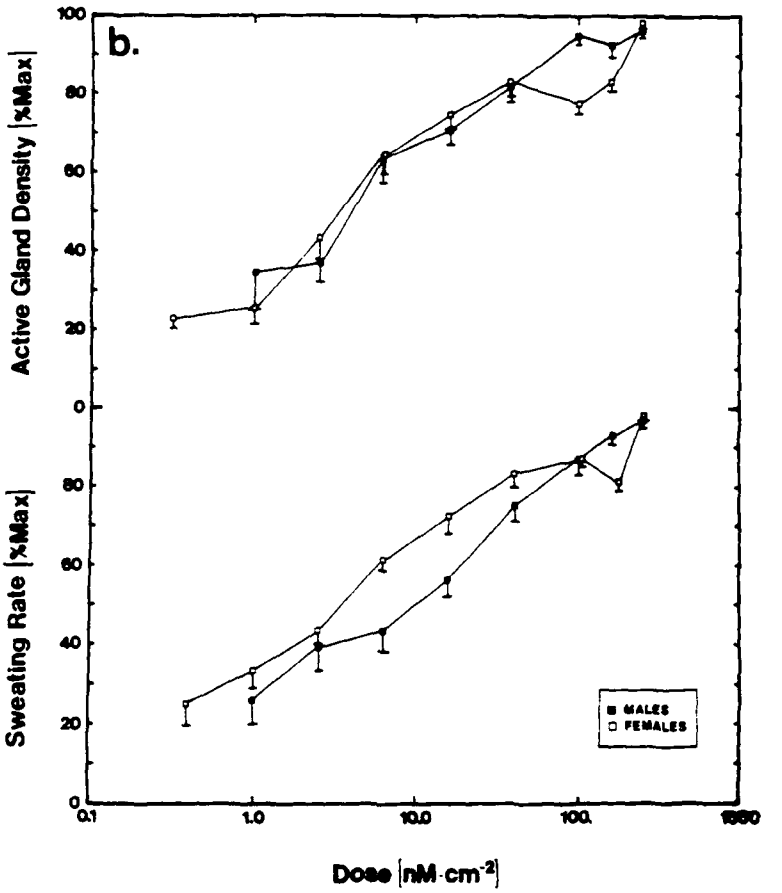

Figure 12. The data of Figure 11 replotted as percent maximal response. This plot shows there is little different in sweat gland recruitment patterns in males and females, but that the sweat glands of females operate at a greater fraction of their maximal capacity at any dose level.

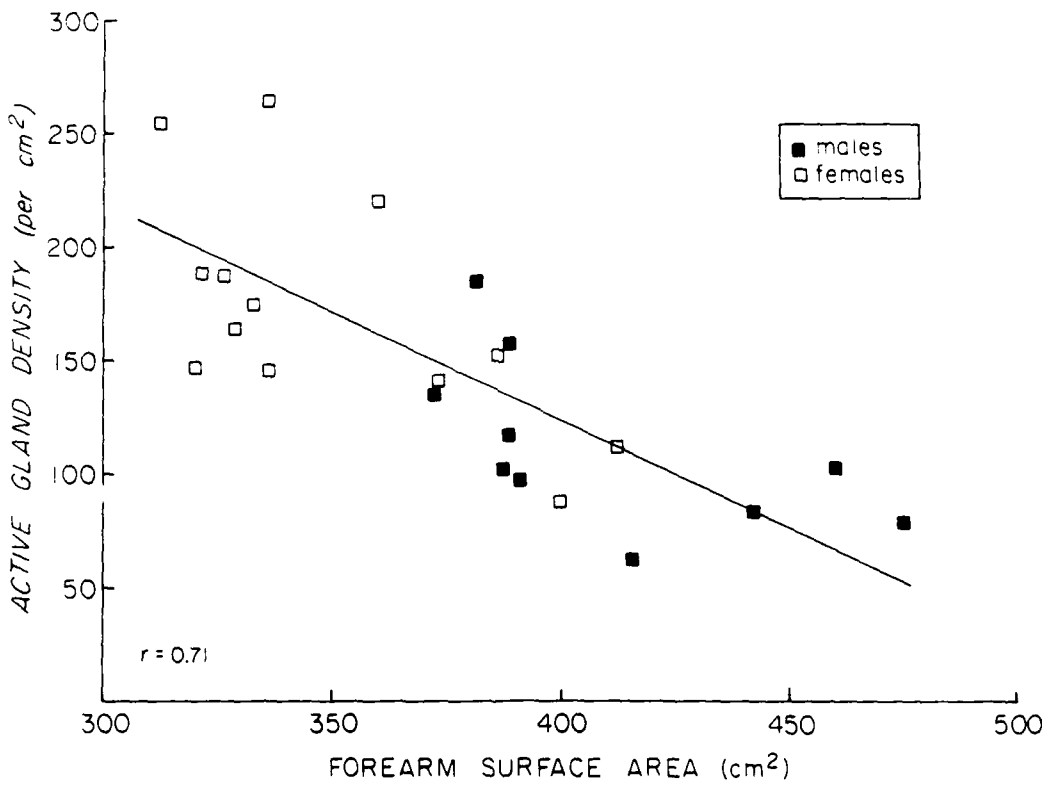

Figure 13. Maximal active gland densities as a function of forearm area (treated as the frustrum of a right cone) under the treatment area. This inverse relationship was significant, showing that persons with smaller forearms (mainly females) had greater gland densities than persons with large forearms (mainly males).

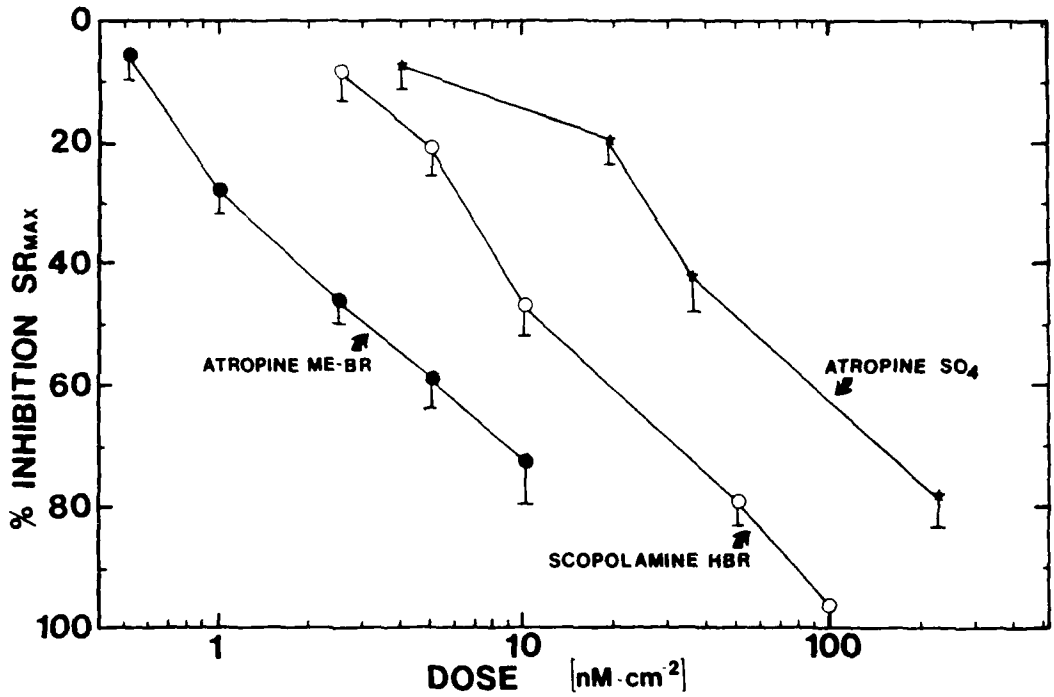

Figure 14. Percent inhibition of maximal sweating rate (from mecholyl iontophoresis) by three anticholinergic agents (administered as iontophoretic pretreatment) in 12 male subjects.

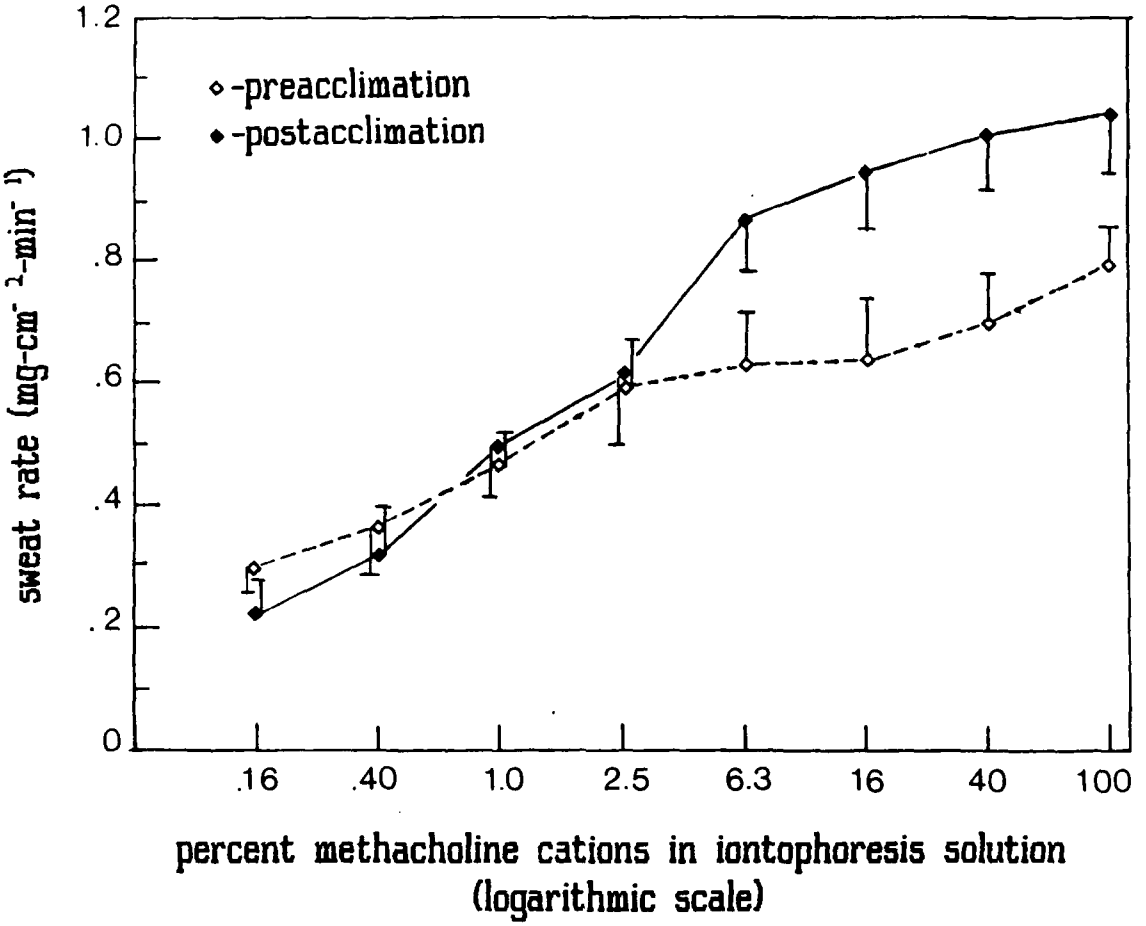

Figure 15. The effect of acclimation on response to the sweat gland assay. A significant increase in sweating rate (average 40%) was observed in all 6 subjects for mecholyl doses greater than 16 nMol-cm^{-2}.

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